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Ecf, an Alternative Sigma Factor from *Neisseria gonorrhoeae*, Controls Expression of *msrAB*, Which Encodes Methionine Sulfoxide Reductase

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A DNA microarray was used to identify genes transcribed in *Neisseria gonorrhoeae* using Ecf, an alternative sigma factor. No differences between the transcriptional profiles of strain FA1090 and a mutant where *ecf* had been inactivated could be detected when both were grown in vitro. We therefore constructed a gonococcal strain in which Ecf can be overexpressed. Some differentially expressed genes are clustered with *ecf* on the genome and appear to form a single transcriptional unit. Expression of the gene encoding MsrAB, which possesses methionine sulfoxide reductase activity, was also dependent on Ecf, suggesting that the regulon responds to oxidative damage. Western blotting confirmed that the increased level of MsrAB protein is dependent on the presence of Ecf.

Bacterial sigma factors are essential components of the RNA polymerase holoenzyme and determine promoter selectivity and specificity. Bacteria usually contain at least one essential sigma factor, sigma-70, which is necessary for cell viability, as well as a number of accessory sigma factors that are often involved in responses to environmental stimuli or the phase of growth. The relative amount of RNA polymerase holoenzyme containing each sigma factor determines the amplitude of the expression of a specific collection of genes.

We have searched the genome sequence of *Neisseria gonorrhoeae* strain FA1090 (GenBank accession number AE004969) for the presence of genes encoding sigma factors. As expected, there is a gene, *rpoD*, encoding sigma-70 (NGO0999). Only two intact genes encoding alternative sigma factors, *rpoH* (NGO0288) and *ecf* (NGO1944), were found. Laskos et al. (11) have shown that there is also an inactive RpoN-like sigma factor, RLS, although this sequence feature (NGO1766) has not been annotated as such in the publicly available annotations of the *N. gonorrhoeae* strain FA1090 genome sequence (GenBank accession number AE004969; annotations at www.stdgen.lanl.gov, cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi, and www.ncbi.nlm.nih.gov/genomes/lproks.cgi). The *ecf* gene encodes a member of the extracytoplasmic function (ECF) family of sigma factors. As the name suggests, ECF sigma factors from different bacterial species appear to respond specifically to a variety of extracytoplasmic stimuli. Characteristically, their activity is controlled by anti-sigma factors, and they control relatively small regulons (1). In most examples of this system investigated to date, the ECF sigma factors regulate not only their own expression but also that of the genes encoding the cognate anti-sigma factor, which are located in the same operon.

We have used DNA microarrays to assess the role of the sigma factor Ecf in gonococci and show that it controls a small regulon which contains the *msrAB* gene, which encodes an unusual methionine sulfoxide reductase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strain DH5α [F^- *endA1 thi-1 hsdR17 supE44 relA1* Δ*lacU169* (ϕ80 Δ*lacZ*M15)] was used for genetic manipulation of constructs. *E. coli* strains were grown in Luria-Bertani (LB) broth (Difco) at 37°C with agitation or on LB agar plates supplemented with 1.5% agar, and where appropriate, with the following antibiotics: 100 μg/ml ampicillin, 25 μg/ml kanamycin, 150 μg/ml erythromycin, or 10 μg/ml tetracycline. Gonococcal strains were grown on GC agar base (Oxoid) or GC broth as described previously (7). Where appropriate, media were supplemented with 7 μg/ml erythromycin, 12.5 μg/ml tetracycline, or 40 μg/ml kanamycin. Transformation and conjugation experiments with *N. gonorrhoeae* were performed as described previously (12) with the exception that conjugation experiments involved mixing 5×10^8 donor cells and 1.5×10^9 recipient cells.

Construction of an *ecf* mutant of *N. gonorrhoeae*. All DNA manipulations were performed by standard methods as described previously (7). Oligonucleotide primers are listed in Table 2. The *ecf* gene from *N. gonorrhoeae* strain FA1090 was amplified using the Expand long-template PCR system (Roche Diagnostics) and primer pair 5673 and 5566 (Table 2). The 2.3-kb PCR product was purified using QIAquick PCR purification spin columns (QIAGEN), treated with T4 DNA polymerase, and ligated to HincII-digested pUC18 (31) to create pJKD2601 (Table 1). The *aphA1* cassette was excised from pUC4K (28) by HincII/EcoRI digestion, gel purified, and treated with T4 DNA polymerase to remove nucleotide overhangs. This purified cassette was ligated into pJKD2601, which was linearized at the HincII site within *ecf* and used to transform *E. coli* DH5α. Ampicillin- and kanamycin-resistant colonies were screened by PCR for the presence of the insert. The resultant plasmid, pJKD2603 (Table 1), was linearized with SphI, and the mutated gene was recombined into the genome of *N. gonorrhoeae* strain FA1090 by homologous recombination. Transformants were isolated by selection for kanamycin resistance, and the presence of the *aphA1* cassette in *ecf* was confirmed by PCR. The *ecf* mutant was designated JKD5069.

Construction of a strain of *N. gonorrhoeae* that can overexpress the *ecf* gene. JKD5062, in which *ecf* can be expressed from an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible P_{trc} promoter, was created in the following manner. The *ecf* gene from *N. gonorrhoeae* strain FA1090 was amplified using oligonucleotide primers 20399 and 20398 (Table 2). Each primer contained a BamHI

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference
Plasmids		
Hermes-8	*E. coli*/*N. gonorrhoeae* shuttle vector, erythromycin resistance	10
pJKD2601	2.3-kb PCR product amplified from the *N. gonorrhoeae* FA1090 genomic DNA with primer pair 5673 and 5566, inserted into HincII-digested pUC18	This study
pJKD2603	*aphA1* cassette ligated into pJKD2601 which was linearized at a HincII site within *ecf*	This study
pJKD2606	0.5-kb PCR product containing the *ecf* gene from *N. gonorrhoeae* strain FA1090 ligated into BamHI digested Hermes-8	This study
pJKD2623	1.7-kb PCR product (containing the *msrAB* promoter region) amplified with primers 23764 and 23763 and ligated to HincII-digested pUC18 vector	This study
pJKD5065	pJKD2606 linearized and recombined into p*tetM*25.2	This study
pJKD5133	Empty Hermes-8 shuttle vector recombined into p*tetM*25.2	This study
p*tetM*25.2	Gonococcal conjugative plasmid	10
pUC18	Cloning vector, ampicillin resistance	31
pUC4K	Cloning vector used as source of *aphA1*(kanamycin resistance)cassette, ampicillin resistance	28
N. gonorrhoeae strains		
FA1090	Wild-type strain	4
JKD484	Spontaneous rifampin-resistant mutant of strain *N. gonorrhoeae* MS11-A, carrying p*tetM*25.2	12
JKD5062	JKD5069(pJKD5065)	This study
JKD5064	JKD5083(pJKD5133)	This study
JKD5065	JKD484 carrying pJKD5065, resulting from recombination between pJKD2606 and p*tetM*25.2	This study
JKD5069	*ecf* mutant of strain FA1090	This study
JKD5083	Spectinomycin-resistant mutant of *N. gonorrhoeae* strain FA1090	This study
JKD5133	JKD5083 carrying Hermes-8 shuttle vector recombined into the p*tetM*25.2 (pJKD5133)	This study

site to assist in cloning, and primer 20399 also contained a modified ribosome binding site for improved translation of the mRNA. The PCR product was gel purified, digested with BamHI, and ligated to a BamHI-digested, alkaline phosphatase-treated Hermes-8 shuttle vector (Table 1). Briefly, this shuttle vector has an *ermC′* gene capable of being expressed in both *E. coli* and *N. gonorrhoeae*, an inducible promoter that is also functional in *N. gonorrhoeae* and is upstream of a multiple cloning site, and the *lacI* repressor gene (10). The initial *E. coli* transformants were isolated on erythromycin plates and screened for the *ecf* insert by PCR. The resultant plasmid, pJKD2606, was linearized with SacII and used to transform *N. gonorrhoeae* strain JKD484, which carries the conjugative plasmid p*tetM*25.2 (10). Transformation of competent *N. gonorrhoeae* strains involves recombination of Hermes constructs into the permissive region of the conjugative plasmid p*tetM*25.2 by allelic replacement (10). Erythromycin- and tetracycline-resistant transformants were tested by PCR to confirm that pJKD2606 had recombined into p*tetM*25.2, and this strain was designated JKD5065 (Table 1). The resulting plasmid, pJKD5065, was transferred into JKD5069 by conjugation. The resulting strain was designated JKD5062 (Table 1). The *ecf* gene in pJKD5065 was sequenced to verify that no mutations had been introduced. A control strain, JKD5064, was constructed by conjugation of pJKD5133 (empty Hermes-8 shuttle vector recombined into the p*tetM*25.2 conjugative plasmid) from JKD5133 into JKD5083 (Table 1).

For investigation of the Ecf regulon, gonococcal strain JKD5062 was grown to mid-exponential phase (optical density at 560 nm = 0.6), and the culture was then split between two prewarmed flasks. One of the cultures was induced with 2 mM IPTG for 30 min. Samples from cultures were harvested and stored in RNA*later* RNA stabilization solution (Ambion). The RNA from these samples was extracted using the RNeasy midi kit (QIAGEN) with an optional on-column DNase I digestion step according to the manufacturer's instructions. The quality and quantity of RNA were determined by gel electrophoresis and spectrophotometry.

cDNA synthesis and fluorescent labeling. Total RNA (30 μg) was mixed with 30 μg of random hexamers, heated to 70°C for 10 min, and then rapidly chilled on ice. To this mixture 0.5 μl (20 units) of RNasin (Promega), 6 μl of Superscript II buffer (Life Technologies, Inc.), 3 μl dithiothreitol (DTT), 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.4 mM dTTP (Promega), 0.6 mM amino allyl-dUTP (Sigma), and 2 μl (400 units) Superscript II reverse transcriptase (Life Technologies, Inc) were added, and the mixture was incubated for 2.5 h at 42°C. Reactions were terminated by the addition of 10 μl of 1 M NaOH and 10 μl of 0.5 M EDTA, and the mixtures were heated to 65°C for 15 min and neutralized by the addition of 25 μl of 1 M Tris, pH 7.4. Unincorporated amino allyl-dUTP was removed with Microcon 30 columns (Millipore) according to the manufacturer's instructions. The purified cDNA was concentrated to 12 μl, and the quality of labeling and quantity of cDNA were determined using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies). Cy3 and Cy5 dyes were dissolved in dimethyl sulfoxide (Sigma) to a final concentration of 14 μg/μl, and a 4-μl aliquot was added to each cDNA sample in 0.1 M sodium bicarbonate buffer (pH 9). Reaction mixtures were incubated for 1 h at room temperature in the dark, labeled cDNA was purified using Microcon columns, and the eluted samples were concentrated to 10 μl using a SpeedVac SVC100 (Savant). Cy3 and Cy5 dye incorporation was estimated by spectrophotometry.

Microarray hybridization and data analysis. The labeled cDNA was hybridized to a pan-*Neisseria* microarray which contains 2,704 PCR products, spotted in triplicate, corresponding to potential coding sequences from *N. gonorrhoeae* strain FA1090, *N. meningitidis* strains MC58 and Z2491, the *N. gonorrhoeae* strain MS11 gonococcal genetic island (8), and various controls. Details of the construction of this array will be published elsewhere, but details and arrays can be obtained from the corresponding author. Prior to prehybridization, the slide was plunged into a 95°C water bath for 2 min, centrifuged for 5 min at 2,000 × *g*, and used immediately. Prehybridization was carried out in a volume of 30 μl containing 25% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), 10 mg/ml bovine serum albumin (BSA; fraction V), and 30 μg of herring sperm DNA (Promega) under a coverslip in a humidified Corning CMT hybridization chamber for 45 min at 42°C. The slide was rinsed in water, dried by centrifugation, and used immediately for hybridization. The labeled cDNA was added to the hybridization fluid (prehybridization solution without BSA) in a total volume of 30 μl and denatured at 95°C for 5 min. The hybridization fluid was placed on the surface of the microarray under a coverslip. The slide was enclosed in the hybridization chamber and submerged in a 42°C water bath overnight. After hybridization, slides were washed once in 2× SSC–0.1% SDS for 5 min at 42°C, once in 0.1× SSC–0.1% SDS for 10 min at room temperature, and four times in 0.1× SSC for 1 min at room temperature. The slides were rinsed with water for 10 seconds, and excess fluid on the surface of the slide was removed by centrifugation before scanning. A GMS 418 array scanner (Genetic Microsystem) was used to scan the images. The Cy3 and Cy5 images were combined, and fluorescent and background intensity for each spot was determined using ImaGene version 5 software (BioDiscovery) as outlined previously (2). Data from poor spots that were manually or automatically flagged in ImaGene were removed from further analysis.

The individual ImaGene data files were uploaded to a web site created with BASE (19) and converted to a common BASE format using a series of custom-made applications. The spot intensities were found to be most reliable when no background correction was performed. Analysis was done using Bioconductor (5) and Limma (23). The normalization of the data to remove various biases involved two steps. Firstly, each array was normalized independently using print-

TABLE 2. Oligonucleotide primers

Oligonucleotide	Sequence (5′–3′)	Reference	Use[a]
3260	CACACTGGGACTGAGACATG	12	Northern blotting; amplification of 16S rRNA probe
3261	CGGCAGTCTCATTAGAGTGC	12	Northern blotting; amplification of 16S rRNA probe
5566	GGCCTGTCCTACGAGTAG	This study	Amplification of *ecf* region
5673	ACCGACCGTTTGGGACTTGCTC	This study	Amplification of *ecf* region
16764	GCTGCCGTCCATTTTCATG	This study	qRT-PCR of *recA*
16766	TGGCGCAAATCGAAAAAAGT	This study	qRT-PCR of *recA*
20398	CGGGATCCCGTTTACTTCGGGTTTTCTTG	This study	Amplification of *ecf* gene
20399	CGGGATCCAGGAGGATACTATGCCGCTACCCGACCTG	This study	Amplification of *ecf* gene
23108	CTTGGCAGACGGCATCCG	This study	Northern blotting; amplification of NGO1948 probe
23110	AAGGCAGCATCAACGAAGCG	This study	Northern blotting; amplification of *msrAB* probe
23111	CATTTTATAACCGTTGTCGC	This study	Northern blotting; amplification of NGO1948 probe, 5′ RACE
23112	TTGCTGCTCGCGTTTGAGGG	This study	Northern blotting; amplification of *msrAB* probe
23488	CGCCGTATGATGCACCATT	This study	qRT-PCR of NGO1946
23489	AACGACCATCAGCCCCAAT	This study	qRT-PCR of NGO1948, 5′ RACE
23494	ACGGCGGTCATCTTTACGAT	This study	qRT-PCR of NGO1946
23495	CACCCTGACCGAAGAGCAAT	This study	qRT-PCR of *msrAB*
23496	TCGTGGCTGAAGGCGTATTC	This study	qRT-PCR of *msrAB*
23497	GCTGCCCGTACTGTTGCTTT	This study	qRT-PCR of NGO1948
23573	TTCCCAAAACGGGCATTG	This study	qRT-PCR of *ecf*
23574	TGATTTTTCCGGAGTGTTCCA	This study	qRT-PCR of *ecf*
23762	ACGTGGATAAAGTGTGCG	This study	Primer extension of *msrAB* tsp
23763	TCTTCGTAGCTCGGGTTTTCC	This study	Amplification of *msrAB* promoter region
23764	CAAGCACGATGTCGATCC	This study	Amplification of *msrAB* promoter region
DT88	GAAGAGAAGGTGGAAATGGCGTTTTGG	27	5′ RACE
DT89	CCAAAACGCCATTTCCACCTTCTCTTC	27	5′ RACE

[a] qRT-PCR, quantitative real-time RT-PCR.

tip loess normalization (Y. H. Yang, S. Dudoit, P. Luu, and T. P. Speed, presented at SPIE BiOS 2001, San Jose, Calif.). Secondly, diagnostic plots suggested a variation in scale between arrays, so the log ratios were scaled in such a way that each array had the same median-absolute-deviation. The normalized data were then used to fit a linear model (23) for each gene using a generalized least-squares method which took into account the correlation between replicate spots (24). The coefficient of the fitted model for each gene describes the inferred difference in RNA expression between the two strains. Empirical Bayes method was then used to calculate the moderated *t* statistics and associated *P* values. The *P* values were adjusted for multiple testing using the false-discovery rate. Genes with an absolute ratio greater than 1.5-fold and significant at the 0.001 level were selected as differentially expressed.

Quantitative real-time reverse transcriptase PCR (RT-PCR). cDNA was generated from 5 μg of the total RNA samples used for the microarray experiments. The RNA preparation was subjected to a second DNase I treatment before cDNA synthesis. The cDNA synthesis was performed as described above with the exception that a 1 mM concentration of each dNTP and 7.8 μg of random hexamers were used. Oligonucleotide primer pairs specific for each gene of interest were designed using ABI PRISM Primer Express software (Applied Biosystems) and are listed in Table 2. To quantitate the cDNA, a gene-specific standard curve method was employed using serial dilutions of strain FA1090 genomic DNA as the template. All assays included 12.5 μl of SYBR Green PCR master mix (Applied Biosystems), 2 μl of each primer (0.5 nM final), 2 μl of template, and diethyl pyrocarbonate-treated water made up to a final volume of 25 μl. Controls lacked reverse transcriptase or template. Reactions were run on an ABI 7700 sequence detection system (Applied Biosystems), and *recA* was used as the normalizer for all reactions. All reactions amplified a single product, as determined by melting curve analysis (Applied Biosystems).

Northern blots. Northern blotting was performed on 10 μg of the total RNA preparations used in the microarray experiments. RNA was electrophoresed on formaldehyde-MOPS (morpholinepropanesulfonic acid) 1% agarose gels and transferred to Hybond N nylon membranes (Amersham) as previously described (20). The transferred RNA was fixed to the nylon membrane with the auto-cross-link setting on a UV Stratalinker 1800 (Stratagene). Probe labeling and detection were performed using a digoxigenin nonradioactive DNA labeling and detection kit (Roche Diagnostics) according to the manufacturer's instructions. Probes were amplified by PCR from each gene using oligonucleotides listed in Table 2.

Identification of transcription start points. Oligonucleotide primer 23762 (Table 2), which is complementary to the *msrAB* sequence, was 5′-end labeled with 30 μCi of [γ-^{32}P]dATP (Amersham) and T4 polynucleotide kinase. Primer extension reactions were performed as described in the instructions for the use of the primer extension system (Promega) using avian myeloblastosis virus reverse transcriptase. The precipitated primer extension products were subjected to electrophoresis in an 8% polyacrylamide gel containing 8 M urea, next to a sequencing ladder generated from plasmid pJKD2623 (Table 1) with oligonucleotide primer 23762 (Table 2). Plasmid pJKD2623 was constructed by amplifying a PCR product encompassing the promoter region of *msrAB* with primers 23764 and 23763 (Table 2), T4 filling, and ligation to HincII-cut pUC18 vector. Primer extension gels were dried onto chromatography paper and subjected to autoradiography.

To confirm the transcription start points, 5′ RACE (rapid amplification of cDNA ends) experiments were performed (27) with the same RNA preparations used in the microarray experiments. cDNA synthesis was performed as described previously (27) using a specific oligonucleotide primer, 23111 (Table 2), located 150 to 200 bp downstream from the initiation codon of NGO1948. Briefly, 5′-phosphorylated, 3′-blocked primer DT88 (27) (Geneworks) (Table 2) was anchored to the 5′ end of the cDNA, and a first-round PCR product was amplified using primer DT89 (Table 2), which was complementary to DT88 (27), and oligonucleotide 23111 (Table 2). A second round of PCR used oligonucleotides DT89 and a nested primer, 23489 (Table 2), that was internal to 23111. Controls with and without reverse transcriptase and T4 RNA ligase were included. Purified PCR product was sequenced to determine the 5′ end of the transcript, which represents the transcription start point.

TABLE 3. Genes up-regulated in strain JKD5062 as a result of overexpression of Ecf

ORF ID[a]	Gene name	Fold change[b]		Proposed function
		Microarray[c]	qRT-PCR	
NGO1944[d]	*ecf*	2.2	5.7	ECF family RNA polymerase sigma factor
NGO1946		1.8	3.7	Conserved hypothetical protein
NGO1947		1.8		Hypothetical periplasmic protein
NGO1948		1.8	4.0	Conserved hypothetical protein (possible membrane protein)
NGO2059	*msrAB*	3.1	5.7	Methionine sulfoxide reductase

[a] ORF ID, identification from the annotation of the GenBank entry (AE004969).
[b] Ratio of mRNA transcript levels in induced relative to uninduced JKD5062.
[c] Expression ratio for genes that were up-regulated 1.5-fold with a P value of <0.001.
[d] Overexpression of a plasmid-borne copy of this gene is driven by an IPTG-inducible P_{trc} promoter. The ratio is a reflection of the engineered levels of transcription of this gene in induced relative to uninduced JKD5062.

Western blots. Western blotting was performed as described previously (11). The samples were prepared from whole-cell extracts of induced and noninduced gonococcal strains JKD5062 and JKD5064 (Table 1) grown in liquid medium. Prior to electrophoresis, 1×10^9 cells were harvested by centrifugation at $5,000 \times g$ at 4°C for 5 min, resuspended in 500 μl of phosphate-buffered saline and SDS sample buffer (0.2 M Tris, 20% glycerol, 25% SDS, 10% β-mercaptoethanol, 0.1% bromophenol blue; pH 6.8), and boiled for 5 min. For equivalent loading, total protein concentration was determined by the microplate procedure using the bicinchoninic acid protein assay kit (Pierce). Primary polyclonal anti-MsrAB rabbit antiserum was used at a 1:20,000 dilution (22). Peroxidase-conjugated anti-rabbit immunoglobulin was used as the secondary antibody (Chemicon) at a 1:3,000 dilution. The Western blot was completed using the protocol provided with the ECL detection reagent kit (Amersham).

RESULTS AND DISCUSSION

The *ecf* gene of *N. gonorrhoeae* strain FA1090 was amplified by PCR, cloned, and insertionally inactivated, and the resultant construct was used to transform strain FA1090, creating the *ecf* mutant JKD5069 (Table 1). Inactivation of the *ecf* gene did not result in any noticeable change in colony morphology or growth rate on solid or in liquid media (data not shown). DNA microarrays were used to assess whether there were any detectable differences in the transcriptome of JKD5069 compared to FA1090, when both strains were in the mid-exponential phase of growth. Despite the fact that a basal level of transcription of the *ecf* gene could be detected in strain FA1090, when the data from two biological repeat experiments (four hybridizations) were combined and analyzed, no genes were found to be differentially expressed in the mutant compared to the wild type at the 1.5-fold level with a P value of less than 0.001. This result suggests that, under the growth conditions used, Ecf activity might be suppressed by an anti-sigma factor, as is the case in other bacterial species (3, 25). It may also be that the microarray experiment was simply not sensitive enough to detect the expected drop in transcription of Ecf-regulated genes, from a low basal level.

In the absence of any knowledge of the physiological signals to which Ecf responds, we reasoned that activation of the regulon could be obtained by overexpression of the sigma factor. To this end we used an IPTG-inducible P_{trc} promoter in the *E. coli*/*N. gonorrhoeae* Hermes shuttle vector system (10), in the background of the *ecf* mutant JKD5069. The strain constructed, JKD5062, allows exclusive and inducible expression of a plasmid-borne copy of the *ecf* gene (see Materials and Methods). We again used microarrays to measure changes in gene expression when the *ecf* gene in strain JKD5062 was induced for 30 min compared to an uninduced control.

Four genes, NGO1946, NGO1947, NGO1948, and NGO2059, were up-regulated along with *ecf* (NGO1944) itself when *ecf* was overexpressed (Table 3). As might be expected with the overexpression of an alternative sigma factor, no down-regulated genes were detected. Quantitative real-time RT-PCR was used to validate the microarray data for three of the up-regulated genes (Table 3). NGO1946, NGO1947, and NGO1948 are annotated as encoding proteins with no known function. The presence of possible transmembrane domains, as predicted by PSORT (15), suggests that NGO1948 might encode an integral membrane protein. A conserved-domain search (13) with the derived amino acid sequence from NGO1948 also revealed the presence of a motif that is found in the DoxD-like family of proteins. DoxD is a subunit of quinone oxidoreductase, which is involved in the oxidation of sulfur (14, 17). NGO1947 is predicted by PSORT (15) to encode a periplasmic protein. Genes encoding proteins closely related to Ecf and the genes NGO1946, NGO1947, and NGO1948 can be found clustered in other bacterial genomes, where the gene order also seems to have been at least partially conserved (data not shown). The expression ratio for NGO1945, the gene located between *ecf* and NGO1946, was just below the 1.5-fold cutoff used in these experiments.

We also observed an up-regulation of the *msrAB* gene (NGO2059), and this was confirmed by real-time RT-PCR (Table 3), suggesting that transcription of this gene is also dependent on Ecf. The protein now termed MsrAB (previously called PilB) appears to be involved in survival in the presence of oxidative damage (22). In addition, it has been recently reported that the expression of *msrAB* in gonococci is induced by exposure to hydrogen peroxide (26). The MsrAB protein is unusual in that it contains three domains with separate enzymatic activities that are often found in different proteins in other bacterial species (6). The carboxy-terminal domain possesses MsrB methionine sulfoxide reductase activity specific for the R isomer of methionine sulfoxide, and a central domain has the complementary MsrA activity with specificity for the S isomer (16). The amino-terminal domain contains a signal sequence and possesses a disulfide reductase activity that can recycle methionine sulfoxide reductases (30). This activity allows recycling of MsrA and MsrB activity in the periplasm, a task usually performed by thioredoxin in other species where methionine sulfoxide reductases are cytoplasmic (6).

One component of the anti-sigma factors controlling ECF activity is an inner membrane protein with at least one transmembrane domain (18). NGO1948 encodes a protein with a

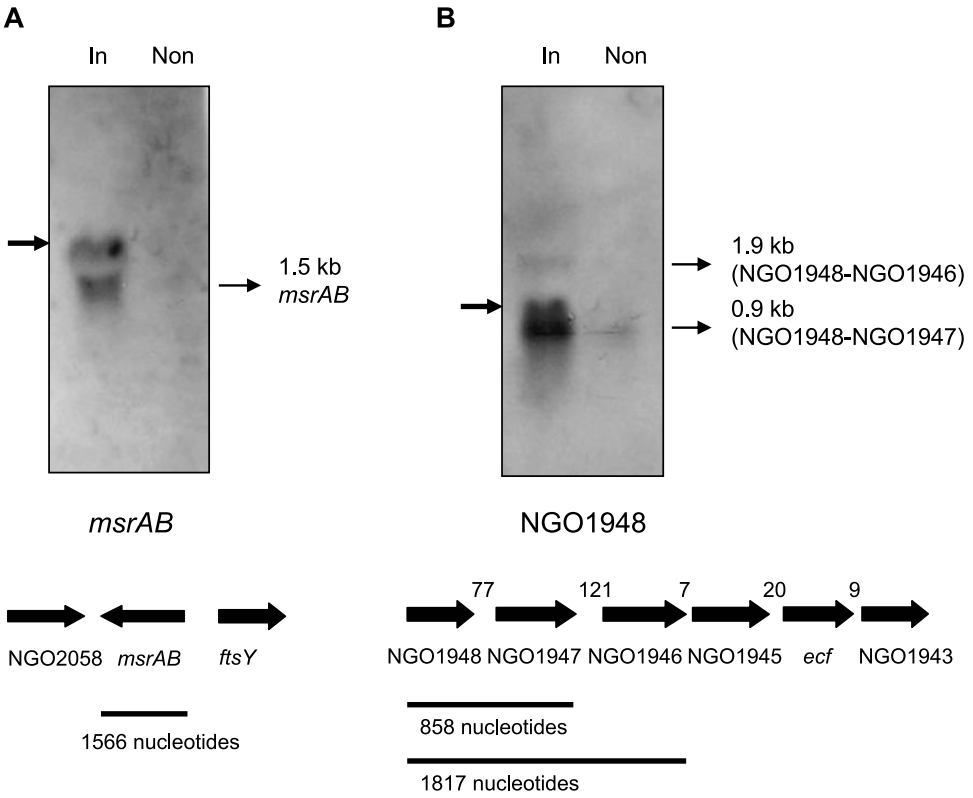

FIG. 1. Detection of *msrAB* and NGO1948 transcripts by Northern hybridization. Total RNA was isolated from *N. gonorrhoeae* strain JKD5062, both with (In) and without (Non) overexpression of *ecf*, and separated on a 1.2% denaturing agarose gel. The separated RNA species were probed with an *msrAB*-specific (A) or an NGO1948-specific (B) probe. The *msrAB*-specific probe detected a 1.5-kb transcript (A, thin arrow). The NGO1948-specific probe detected two transcripts (B, thin arrows) of 0.9 kb and 1.9 kb. Nonspecific bands (bold arrows) were artifacts comigrating with rRNA. Below the gels are diagrammatic representations of the putative genes in the region, with approximate sizes of transcripts (B), in nucleotides.

predicted transmembrane domain. However, as is the case with the sigma E proteins from *E. coli* and *Streptomyces coelicolor*, multiple genes can have a role in the anti-sigma function (3, 29). Taking into account the observation that Ecf appears to be relatively inactive in strain FA1090 during the exponential phase of growth, the possibility exists that NGO1946 to NGO1948 encode proteins that function to inhibit Ecf activity. In addition, the genomic arrangement of NGO1944 (*ecf*), NGO1945, NGO1946, NGO1947, and NGO1948 suggests the possibility of cotranscription of at least some of these genes. Genes encoding several ECF sigma factors have been shown to be cotranscribed with a gene(s) encoding a specific anti-sigma factor(s) (3, 9, 21, 25). Northern hybridization was performed with total RNA isolated from strain JKD5062, with or without induction of *ecf*. A strong signal was observed from RNA prepared after induction of *ecf* with probes derived from both *msrAB* and NGO1948, suggesting the presence of Ecf-controlled promoters upstream of these genes (Fig. 1). The probe specific for NGO1948 hybridized to a major 0.9-kb band and a minor 1.9-kb mRNA transcript. The presence of the latter suggests that at least NGO1946, NGO1947, and NGO1948 can be cotranscribed. Larger transcripts are unlikely to have been detected in this experiment. The major 0.9-kb transcript corresponds in nucleotide length to NGO1948 and NGO1947, which suggests the presence of a transcriptional attenuator at the end of NGO1947. An inverted repeat sequence can be found in this intergenic region (data not shown). The minimal spacing between the remaining genes in the cluster (Fig. 1) suggests that they may be cotranscribed. An inverted repeat sequence that may act as a terminator is found downstream of NGO1943. With this strain, we were unable to look directly for cotranscription of the other genes with *ecf*, as this gene is transcribed from the multicopy vector in strain JKD5062. As expected, given the orientation of the genes neighboring *msrAB* (Fig. 1), this probe hybridized to a band of the correct size for a single gene transcript.

Each ECF sigma factor, even within a bacterial species, recognizes specific promoter sequences. The promoters upstream of *msrAB* and NGO1948 were mapped using primer extension analysis and 5′ RACE. The transcription start point (TSP) of *msrAB* was mapped to an A residue located 25 nucleotides upstream of the putative translational start site (Fig. 2A), and this was confirmed using 5′ RACE (data not shown). This TSP was observed only when *ecf* was overexpressed (Fig. 2A). We were unable to identify a TSP for NGO1948 using primer extensions, but 5′ RACE revealed a TSP at an A residue (Fig. 2B) that again was observed in RNA samples only when *ecf* was overexpressed (data not shown). Examination of regions upstream of the NGO1948 and *msrAB* TSP revealed a consensus sequence apparently recognized by Ecf (Fig. 2C). This included a region around the −35 box where five of the eight bases were conserved, but this sequence

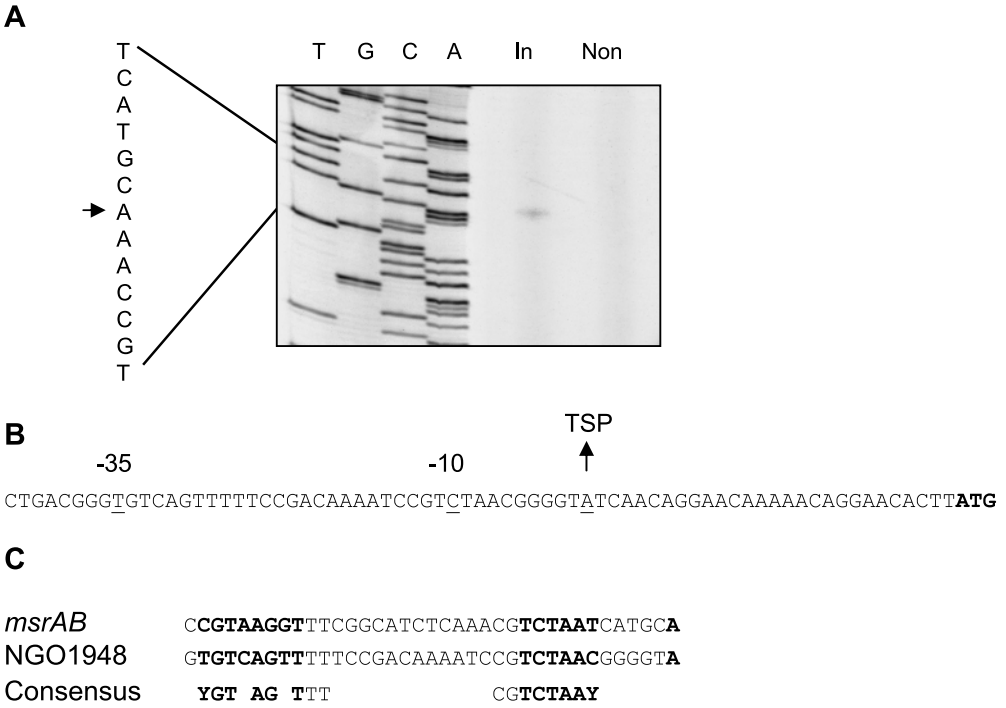

FIG. 2. Identification of transcription start points of Ecf-regulated genes. (A) Primer extension mapping of the *msrAB* TSP. The 5′ terminus of the *msrAB* transcript was determined using RNA prepared from cultures induced for *ecf* overexpression (In) and noninduced cultures (Non). The lanes marked T, G, C, and A are the sequencing ladder that was generated with the primer used for primer extension and pJKD2623 (Table 1), which contains the putative promoter. In the sequence on the left, the arrow indicates the transcription start point. (B) 5′ RACE mapping of the NGO1948 TSP. The sequence upstream of NGO1948 is shown; the start codon for the putative gene is in bold. (C) Alignment of the promoter regions of NGO1948 and *msrAB* with the putative *ecf* consensus sequence shown below. The −10 and −35 regions are in bold.

did not contain the common AAC motif found in this region of most ECF-regulated promoters (9). In addition, a well-conserved AT-rich region was identified 16 bases downstream around the −10 region. Using this consensus sequence, we searched the *N. gonorrhoeae* strain FA1090 genome sequence using FUZZNUC (http://psychro.bioinformatics.unsw.edu.au) but did not identify any additional genes that might belong to the Ecf regulon by this method.

We investigated expression of the gonococcal MsrAB protein to ascertain whether the observed increase in transcription of *msrAB* translated into increased protein levels. Western blot analysis using an anti-MsrAB polyclonal antibody was performed on whole-cell extracts prepared from strains JKD5062 and JKD5064 (Table 1), which carries an empty vector, grown to mid-exponential phase and harvested with and without induction for 30 min and 60 min. The gonococcal *msrAB* gene (NGO2059) appears to be responsible for the production of two proteins. In addition to the full-length, three-domain, secreted protein, an internal initiation codon and ribosome binding site allow the translation of a truncated, two-domain cytoplasmic protein with MsrA and MsrB activity (6, 22). Only the secreted form of MsrAB appears to be involved in resistance to oxidative damage (22). JKD5062 induced for 60 min produced a protein of approximately the same size as the full-length MsrAB, suggesting that the transcriptional increase results in elevated levels of MsrAB protein (Fig. 3). However, the majority of the protein detected at both time points appeared to correspond to a truncated form of MsrAB, similar in size to that observed by others (22) using the same antiserum. The antiserum did not detect any protein from JKD5064, reinforcing the finding of dependence on Ecf for expression of *msrAB*.

In summary, this work has identified a regulon for the only ECF sigma factor of *N. gonorrhoeae* and a potential consensus sequence for promoters recognized by this sigma factor. This regulon could confer increased protection against oxidative damage. This suggestion is strengthened by the observation

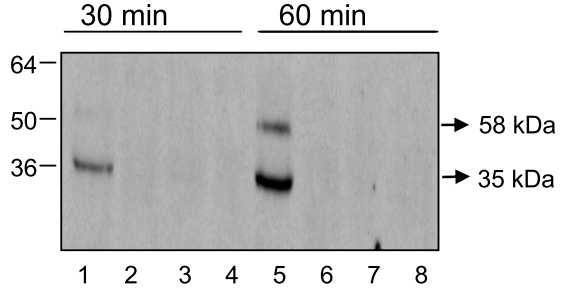

FIG. 3. Western blot of cell extracts from *N. gonorrhoeae* using a polyclonal antiserum to detect MsrAB. Marker protein sizes are in kDa. Lanes 1, 3, 5, and 7 contain extracts from *ecf* overexpression strain JKD5062, while lanes 2, 4, 6, and 8 contain extracts from control strain JKD5064, carrying an empty vector. Lanes 1, 2, 5, and 6 contain extracts from cultures that were induced with IPTG, while lanes 3, 4, 7, and 8 contain extracts from noninduced cultures. Lanes 1 to 4 contain extracts that were sampled after 30 min, while lanes 5 to 8 contain extracts that were sampled after 60 min.

that three genes in the ECF regulon (NGO1947, NGO1948 and *msrAB*) were up-regulated during exposure of gonococci to hydrogen peroxide (26). In addition, this is the first work to describe a regulatory network controlling the expression of *msr* genes in bacteria.

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